Bull. Org. mond. Santé
Bull. Wld Hlth Org. } 1959, **20**, 411-420

METHODS AND PROCEDURES FOR USE OF COMPLEMENT-FIXATION TECHNIQUE IN TYPE- AND STRAIN-SPECIFIC DIAGNOSIS OF INFLUENZA *,†

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SYNOPSIS

It is now well established that there are two kinds of complement-fixing antigens of influenza virus: the soluble, type-specific, antigens, found mainly in infected tissue but also within the virus particle; and the virus, strain-specific, antigens, closely linked with the haemagglutinating component. For true results in complement-fixation testing in influenza, antigens or antisera of the one kind must be free of the other kind. This paper describes in detail the preparation of reagents for such testing and a variety of basic aspects of test procedures.

The existence of two kinds of complement-fixing antigens of influenza virus is well established. The soluble (S) antigen is predominantly found in infected tissues (free S) but it is also present within the virus particle (internal S) (Hoyle, 1952; Lief & Henle, 1956a). The virus (V) antigen is closely linked with the haemagglutinating component, if not identical with it. The S antigens are type-specific (Lennette & Horsfall, 1941; Lief, Fabiyi & Henle, 1958); i.e., they are identical for all strains of one type. The V antigens are strain-specific (Fabiyi, Lief & Henle, 1958); i.e., they differ to a greater or lesser extent depending upon the year of isolation of the strain, in analogy with the differences measured by the haemagglutination-inhibition technique.

In order to obtain valid results in complement-fixation tests concerning various aspects of diagnosis it is obviously essential to prepare reliable reagents; that is, V antigens free of S and S antigens devoid of V and,

* The techniques described have been developed under a grant-in-aid from the National Institutes of Health, United States Public Health Service.

† This paper is also published as an annex te the report of the WHO Expert Committee on Respiratory Virus Diseases (*Wld Hlth Org. techn. Rep. Ser.*, 1959, 170).

correspondingly, anti-V sera lacking anti-S and anti-S sera without anti-V. It is now possible to produce these reagents by relatively simple means (Lief & Henle, 1956a, 1956b; Lief, Fabiyi & Henle, 1958; Fabiyi, Lief & Henle, 1958). The specific sera are helpful in the rapid identification of new isolates of influenza virus as to type (Sigel et al., 1949), and in the determination of antigenic patterns of strains (Fabiyi, Lief & Henle, 1958; Lief et al., 1959). Pure S and V antigens are of advantage in the type- and strain-specific serodiagnosis of the disease (Henle, Lief & Fabiyi, 1958).

In the following sections the methods of preparation of the reagents and their application to various diagnostic problems will be presented.

Preparation of Reagents

V antigens

For *in vitro* antigens, virus particles, separated from free or external S, are satisfactory (Lief & Henle, 1956b). The internal S is not available for interaction with antibodies. For the production of anti-V sera in guinea-pigs, however, it is preferable to employ V antigens from which the internal S has been removed (Lief & Henle, 1956a).

V antigens for in vitro *tests.*

For this purpose suspensions of virus particles are used which are derived from infected allantoic fluids by two cycles of adsorption on to and elution from chicken red cells. Bloody allantoic fluids are harvested from infected chick-embryos shortly after removal from the incubator by tearing the allantoic membranes and permitting free bleeding into the allantoic cavity. The bloody fluids are then immediately placed in an ice-bath for one hour to allow for adsorption of the virus on to the red cells. As an alternative, clear allantoic fluids may be harvested from chilled eggs and washed and packed erythrocytes added to the ice-cold fluids to form a 5% suspension. After the adsorption period the cells are sedimented by centrifugation in the cold, washed once with ice-cold M/100 phosphate-buffered saline solution at a pH of 7.0 (BSS) and resuspended in BSS containing 10 units of receptor-destroying enzyme (RDE), 500 units of penicillin and 100 μg of streptomycin per ml. For each egg harvested 1 ml of BSS is employed. Elution of the virus from the red cells is permitted to take place in a water-bath at 37°C for 2-2½ hours. After removal of the red cells the eluates, constituting sixfold to eightfold concentrations of the virus, are subjected to second cycles of adsorption and elution as described, except that the volumes of BSS used for resuspension of the cells should be adjusted so that the final eluates will contain a minimum of 2560 haemagglutinating (HA) units per ml. For determination of the HA units, 0.4 ml of virus dilution is mixed with 0.2 ml of a 1% suspension of chicken red cells. The

HA titre so obtained, multiplied by 2.5, yields the number of HA units per ml. If other proportions of virus and red cells are employed in the HA test, one HA unit may represent different amounts of virus and the unitage needed for satisfactory V antigen preparations would have to be adjusted accordingly.

The V antigens so obtained are generally free of detectable S (Lief & Henle, 1956b). If found, a third cycle of adsorption on to and elution from red cells is required for its removal.

Vantigens for immunization of guinea-pigs

Virus suspensions obtained after one cycle of adsorption on to and elution from red cells (see above), and containing at least 5120 HA units/ml, are mixed at room temperature with half a volume of anaesthetic ether and agitated continuously for one hour on a magnetic stirrer so that the ether is kept well interspersed with the virus suspension (Lief & Henle, 1956a). The mixtures are then transferred to separating funnels and the aqueous phases are withdrawn. Residual ether is best removed by bubbling nitrogen through the materials. The treatment with ether liberates the internal S, and the HA components may now be separated from the S fraction by adsorption on to and elution from chicken red cells according to the technique described above. The treatment with ether reduces the HA activity of certain strains when tested with chicken red cells, whereas agglutination of guinea-pig erythrocytes is usually increased by a factor of 4. In these cases, the use of guinea-pig instead of chicken red cells is recommended for better yields. A second cycle of adsorption and elution is usually needed to free the HA fractions of detectable S antigen. This is best carried out in the absence of RDE, since on immunization with the eluates guinea-pigs may on occasion form antibodies to this enzyme. In this case the elution period has to be prolonged to up to 6 hours at 37°C in order to recover most of the HA components.

A few virus particles may escape the action of ether and remain infectious. It is recommended, therefore, that the preparation be exposed to ultraviolet light in order to ensure complete inactivation, since an infectious process may result in production of some anti-S. Irradiation of the material in 15-20 ml volumes in open Petri dishes under constant slight agitation for three minutes by a General Electric Germicidal Lamp (or its equivalent) at a distance of 7 inches, or about 18 cm, has been found satisfactory for this purpose. Before use, the preparations are tested for the presence of infectious virus by two blind passages in chick-embryos.

S antigens

S antigen may be derived from infected allantoic membranes or fluids (free S) or from virus particles (internal S). The richest source of S antigen

is infected chorio-allantoic membrane. While all three kinds of preparation are satisfactory for *in vitro* tests, for immunization of guinea-pigs internal S is preferred because of its relative purity.

S antigens for in vitro *tests*

Infected chorio-allantoic membranes. These are collected 24-48 hours after inoculation of the chick-embryos, the optimal time of harvest depending upon the dose of virus inoculated (Kirber & Henle, 1950). A 20%-40% suspension of the tissue in BSS, clarified by low-speed centrifugation, contains as a rule S antigen in excess of V and thus may be used for serological tests. However, in order to avoid all possible interaction of the V antigen also present, it is desirable to remove all HA activity by high-speed centrifugation or exhaustive adsorption of the suspension with chicken erythrocytes, or by both processes.

Infected allantoic fluids. Such fluids, particularly when collected 48-72 hours after inoculation of the eggs, also contain free S (Kirber & Henle, 1950). In this case, the V antigens exceed, as a rule, the concentration of S. Such preparations may serve as reliable S antigens only when the virus particles are removed by high-speed centrifugation or exhaustive adsorption with chicken red cells.

Internal S antigen. Internal S antigen is liberated from virus particles by exposure to ether as described earlier. In this case, adsorption with chicken red cells is often not sufficient because as a result of ether treatment the affinity of the haemagglutinating components for chicken red cells may be reduced, whereas that for guinea-pig erythrocytes is generally increased (Lief & Henle, 1956a). For this reason S preparations derived from certain strains of virus may require adsorption with guinea-pig red cells in order to remove all V antigen.

S antigen for immunization of guinea-pigs

For this purpose S antigen is employed as an intra-abdominal booster injection following intranasal inoculation of active virus (see below). Any one of the S preparations described above exerts the desired booster effect, but in order to minimize anti-V responses the S preparations must be exhaustively adsorbed with red cells. The internal S antigen, liberated from virus particles by ether, constitutes the purest and therefore the most desirable preparation for this purpose.

Anti-V sera

Guinea-pigs are injected intra-abdominally with the HA fraction derived from ether-treated virus particles which have been proved to be

non-infectious by two consecutive blind passages in chick-embryos. The preparation should contain 2560-5120 HA units per ml as measured with chicken red cells, or when this activity has been affected by ether, with guinea-pig red cells. Three doses of 1 ml each are usually sufficient to evoke a satisfactory anti-V response. If preparations of lesser HA activity are the only ones available larger volumes may be injected. The first two doses are best given 5-7 days apart, followed by the third dose 3 weeks later. The sera collected one week after the last injection generally reveal high anti-V titres (1:64 or greater) and no anti-S (<1:4) (Fabiyi, Lief & Henle, 1958). Rare sera showing slight anti-S activity are discarded. Occasionally a fourth dose may be required to elicit satisfactory antibody responses but further injections, as a rule, fail to improve the results. Other dosage schedules, such as three injections at 4-5-day intervals and bleeding 12-18 days later, have also given suitable anti-V responses. The sera are inactivated at 56°C for 30 minutes, absorbed with sheep cells and stored preferably in the frozen state.

A few animals may form antibodies to RDE present in the final eluate employed for immunization. This is more evident when volumes in excess of 1 ml are injected. This response is eliminated by avoiding the use of RDE in the final elution procedure.

Anti-S sera

Guinea-pigs are inoculated under light ether anesthesia with infected allantoic fluid, diluted so as to contain about 10^6 or fewer chick-embryo-infectious doses, 0.1 ml being dropped into each nostril. After 5-8 weeks, when the antibodies evoked by the infection, especially the anti-V, are expected to have decreased to subdetectable levels, an intra-abdominal injection of 1 ml of S antigen is administered. The antigen preferably to be used for the booster dose is the internal S antigen derived from virus particles by exposure to ether. If the intranasal inoculum consists of virus of one subtype (e.g., an A1 strain) and the S antigen is derived from another subtype (e.g., an A strain) only the antibodies to the common S antigen are boosted. Under these conditions about half the animals will reveal 7-10 days after the intra-abdominal injection anti-S titres of 1:64 or greater and no detectable anti-V (Lief, Fabiyi & Henle, 1958). Any V antigen present in the S preparation is generally insufficient to stimulate homologous anti-V and incapable of recalling the heterologous anti-V of the strain used for infection. The sera are inactivated and stored as described above.

If the strains used for the intranasal infection and for preparation of the S antigen for the booster injection are identical or closely related, the sera obtained reveal both anti-V and anti-S in high titres (Lief, Fabiyi & Henle, 1958). Such sera can be used for detection of S antigen in heterologous, homotypic virus preparations as long as the anti-V in the serum bears no relationship to the V antigen under test.

Test Procedures

Numerous modifications of the complement-fixation test are in practice in different laboratories. As long as the standardization procedures for the various reagents are rigid there is no particular reason to prefer one or the other method. For this reason no technical details concerning the test itself will be given below and the presentation will be restricted essentially to principles which should be part of all methods.

Standardization of complement

Since the various viral preparations derived from chick-embryos often exhibit pro-complementary activity (or on occasion anti-complementary effects), titration of complement should be performed always in the presence of representative antigens in the test dose to be employed. This will ensure accurate dosage of complement for the various antigens under test, whether two full units or any other unitage is desired.

Identification of isolates as to type and antigenic patterns

Tests for potency and specificity of anti-V and anti-S sera.

Since zone phenomena are common in the interaction of influenza antigens with their respective antibodies, the sera to be used for identification of isolates have to be assayed for potency and specificity in block titrations, also referred to as two-way, box, or optimal titrations. Duplicate sets of falling twofold dilutions of serum are tested against serial twofold dilutions of (*a*) the homologous V and (*b*) the homotypic S antigens (Lief, Fabiyi & Henle, 1958; Fabiyi, Lief & Henle, 1958). A suitable anti-V serum should react in high titre (1 : 64 or more) with the homologous V antigen but not at all with S, and the anti-S serum should react in high titre with homotypic S but not at all with the V antigens of the strains involved in the production of the anti-S sera.

Standardization of antigens

Reference antigens. The block-titration technique described above serves to standardize the reference V and S antigens with respect to their purity and potency. The optimal unit of V or S antigen is the highest dilution of antigen giving maximal homologous serum titres. It should be noted that the unit determined with appropriate human sera may require twice the amount of antigen measured with guinea-pig sera.

New isolates. It is not always feasible to prepare V antigens from new isolates according to the method described above for *in vitro* tests and to standardize them by the same techniques as reference antigens. The delay

caused by their preparation and in obtaining specific antisera to them is particularly contra-indicated in times of an epidemic. Fortunately, for purposes of strain identification, antigens may be used which contain S (Lief, Ostapiak, Fabiyi & Henle, 1958). The S present will not interfere in the reaction provided the anti-V sera used for assay are free of anti-S. Thus infected allantoic or amniotic fluids may serve as V antigens. In the absence of specific antisera, the V unitage of either elementary body suspensions or infected allantoic fluids may be estimated from the HA titres, since one V antigen unit corresponds to 16 HA units under the conditions of testing referred to above (Lief & Henle, 1956a).

Determination of type of isolates

For this purpose allantoic or amniotic membrane suspensions of HA-positive eggs in the first few passages form a better source of S than the respective fluids, unless the HA titres of these are so high as to suggest that S antigen may also be present in detectable quantities. Suspensions of the tissues, prepared as described earlier, are tested against 4 or more antibody units of anti-S sera of the various types. Known S antigens serve as controls for the sera (Sigel et al., 1949).

Determination of antigenic composition of isolates

The techniques to be employed vary depending upon whether one deals with a member of an established subtype, which has been in circulation for several years, or with a new antigenic variant, representing the first member of a new subtype (Lief, Ostapiak, Fabiyi & Henle, 1958).

Strains of an established subtype

The new isolate, either in the form of infected allantoic fluid or, if possible, in the form of a suspension of virus particles obtained by two cycles of adsorption on to and elution from red cells, is first screened against various anti-V sera prepared with prototype strains of homotypic viruses. The sera are used in dilutions of 1 : 4 or 1 : 8 and the dose of antigen should represent preferably 8 or more V units; i.e., 128 or more HA units per test volume The sera giving a positive complement-fixation reaction are then titrated in falling twofold dilutions against the isolate antigen. The titres obtained are compared with the titres of the anti-V sera with their homologous antigens. If one of the anti-sera reveals similar titres with the isolate and its homologous V antigen, the new agent is assumed to be closely related with respect to the dominant antigens of the strain employed for production of that serum. The incidence and degree of cross-reactions of these two viruses with other anti-V sera will indicate how closely they resemble each other with respect to minor antigenic components. If none of the re-

active sera reveal matching titres with the isolate and their homologous V antigen some change in the dominant antigen must have occurred and antisera to the new virus should be prepared to evaluate the extent of that change.

If none of the anti-V sera show a reaction, yet the type was clearly established, a new subtype may have arisen and such isolates should receive therefore immediate, intensive study in an appropriate laboratory.

New subtype

With the advent of a new subtype, such as the recent appearance of A2, the identification in the first major epidemic can be restricted to a screening procedure, using infected allantoic or amniotic fluid as source of antigen and anti-V sera against prototype viruses (Lief, Ostapiak, Fabiyi & Henle, 1958). The anti-V sera may be used in a dilution containing 4 homologous anti-V units in order to avoid the rare, insignificant cross-reactions that might occur between members of two subtypes. In this fashion only the serum to the new subtype will give a positive reaction. If the HA titre of the virus preparation is low, as often experienced in early passages, the volume tested in the complement-fixation test may be increased up to sixfold without interfering with the reaction. Thus, if the usual volume of antigen employed is 0.1 ml, up to 0.6 can be used without changes in the volumes of the other reagents. The test may then be read after settling of the red cells. Absence of any haemolysis serves to identify the positive reaction. Under such conditions, an allantoic fluid having as few as 30-40 HA units per ml can be used, since the increased volumes will still contain 16 units, which corresponds to one V unit.

Serodiagnosis

The serological diagnosis of influenza during an epidemic, once the offending strain has been established, may well be restricted to the use of S antigen of the corresponding type. However, under certain conditions, such as the appearance of a new subtype, strain-specific serological tests may be of considerable aid. In the past these were restricted to haemagglutination-inhibition tests but now the complement-fixation technique may also be employed using strain-specific V antigens.

For standardization of V and S antigens and reference anti-V and anti-S sera, the reader is referred above to the section on identification of isolates as to type and antigenic patterns.

Type-specific serodiagnosis

For this purpose acute stage and convalescent sera are titrated against 2-4 optimal dilutions of S antigen. A fourfold or greater rise in anti-S

of one type is considered evidence of infection with a strain of that type. Anti-S responses are the rule except in very young children (under two years of age), where no anti-S may be found.

Note. In many laboratories allantoic fluid infected with any one strain of given types or crude allantoic membrane suspensions have been used for serodiagnosis with apparently satisfactory results. However, both types of preparation also contain V antigen. In the allantoic fluids, V antigen is generally found in excess of S, and in the membranes the reverse is true. It is likely that the allantoic fluid antigens, even if old strains are employed, detect essentially a recall of antibodies to V antigens which may be present in minor or even subdetectable concentrations in the infecting virus. The diagnosis consequently might be missed in persons who had few previous experiences with influenza (younger age-groups) and in individuals who already possessed that particular anti-V prior to illness and in whom the stimulus provided by the infection was insufficient to increase the titre.

With the crude membrane suspensions it is likely that mainly anti-S responses are measured but the low levels of V, if not diluted out by restriction of S to 2 optimal units, may interfere with arriving at a diagnosis under the conditions described above.

For these reasons the S antigens for *in vitro* tests described above would seem to be preferable, particularly since the additional step in their preparation, i.e., their adsorption with chicken red cells for the removal of V, is simple and feasible in any laboratory.

Strain-specific serodiagnosis [1]

For this purpose V antigen, preferably of the current strain, or at least a close relative, is required. This is prepared according to the method described above for *in vitro* tests. The acute and convalescent sera are titrated against 2-4 optimal units. If the acute-stage serum is obtained early enough a significant rise in antibodies may be seen in the convalescent sera of practically all patients, including infants, where anti-S responses may not be elicited (Henle, Lief & Fabiyi, 1958). In the case of a first infection with a new subtype, such as A2, the anti-V response may be delayed and convalescent sera obtained 10-14 days after onset may still be negative, whereas later bleedings yield high titres of antibodies.

As is known from experience with the haemagglutination-inhibition test, antibodies to predominant V antigens of other strains may also frequently rise (Henle, Lief & Fabiyi, 1958). These represent a recall of antibodies, elicited by the booster effect of earlier antigens present in the infecting virus in minor or even undetectable concentrations.

The use of the " current " V antigen in serodiagnosis has the following advantages: nearly all patients respond with anti-V, usually from $<1:4$ in the early acute stage to $>1:64$ in convalescent stages; it may still reveal a diagnostically significant rise in antibodies when the acute stage serum is taken relatively late after onset so that anti-S is already high as a result of a recall and does not significantly increase thereafter; and it reveals an antibody response in infants where anti-S may fail to develop.

[1] This subject will be further discussed in two papers now in preparation by the present authors—one on the use of the strain-specific complement-fixation test in the serodiagnosis of epidemic influenza, and the other on the complement-fixation test in evaluating responses to influenza vaccines.

Strain-specific antibody responses following vaccination[1]

The techniques employed for strain-specific serodiagnosis can be applied to assay of anti-V responses following vaccination. The results obtained thus far are comparable to those of the haemagglutination-inhibition technique.

RÉSUMÉ

Il existe dans le virus grippal deux antigènes fixateurs du complément: l'antigène soluble (S) que l'on trouve surtout dans les tissus infectés, mais aussi dans la particule de virus (S interne), et l'antigène viral (V) étroitement lié au composant responsable de l'hémagglutination. L'antigène S est spécifique de type (il est identique pour toutes les souches d'un même type). L'antigène S est spécifique de souche. Il peut varier selon l'année d'isolement de la souche, c'est ce qu'indiquent les tests d'inhibition de l'hémagglutination.

Pour obtenir des résultats dignes de foi dans l'épreuve de fixation du complément, il convient de préparer les réactifs de façon que les antigènes V ne contiennent pas de S, et vice-versa, et que les antisérums V soient dépourvus d'anti-S, etc. Il est maintenant facile de préparer ces divers réactifs. Les antigènes purs S et V sont utiles dans le diagnostic de type et de souche, du virus. Les sérums spécifiques sont employés pour typer les virus récemment isolés et établir le schéma antigénique des souches.

Les auteurs décrivent la préparation des antigènes S et celle des antigènes V pour les tests *in vitro* et pour l'immunisation des cobayes, les procédés d'immunisation de ces derniers, les techniques de fixation du complément et celles de l'identification des types et de la détermination du schéma antigénique des virus nouvellement isolés. Le sérodiagnostic spécifique de type et de souche au cours d'une épidémie est également discuté. Les techniques de sérodiagnostic spécifique de souche peuvent être appliquées à l'étude des réponses anti-V postvaccinales. Elles ont donné jusqu'à maintenant des résultats comparables à ceux du test d'inhibition de l'hémagglutination.

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